

# Functional activity and transmembrane signaling mechanisms after cryopreservation of human internal mammary arteries

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**Purpose:** Cryopreserved human blood vessels are important tools in bypass surgery. However, several in vitro studies have demonstrated diminished postthaw functional activity. Therefore the aim of this study was to investigate the consequences of various freezing/thawing protocols and the role of protein kinase C in the postthaw functional activity of cryopreserved human arteries.

**Methods:** In vitro responses of frozen/thawed human internal mammary arteries (IMA) were used to investigate the functional activity after thawing at 15°, 30°, and 100° C/min and after different prefreezing equilibration times (10, 60, 120, 240 minutes) with the cryomedium (Krebs-Henseleit solution containing 1.8 mol/L dimethyl sulfoxide and 0.1 mol/L sucrose) at room temperature followed by cryo-storage at -196° C.

**Results:** Prefreezing equilibration for 10 to 120 minutes diminished maximal  $\alpha$ -adrenoceptor-mediated responses to noradrenaline to approximately 60%, and equilibration for 240 minutes attenuated noradrenaline effects to less than 25% of that produced by unfrozen controls. Contractile responses were slightly better when thawing was performed at 15° C/min compared with 100° C/min. The postthaw sensitivity to direct activation of protein kinase C by phorbol 12,13-dibutyrate was enhanced. Compared with unfrozen tissues ( $pD_2 = 7.36 \pm 0.07$ ,  $n = 32$ ) maximal sensitization to phorbol 12,13-dibutyrate was observed in IMA that had been frozen after 60 minutes of equilibration with the cryomedium ( $pD_2 = 8.31 \pm 0.09$ ,  $n = 30$ ). Responses to phorbol 12,13-dibutyrate of cryopreserved IMA were highly susceptible to blockade of calcium influx by nifedipine, whereas those of unfrozen IMA were resistant to nifedipine. Against noradrenaline nifedipine was equipotent in cryopreserved ( $pD'_2 = 7.75 \pm 0.15$ ,  $n = 8$ ) and unfrozen IMA ( $pD'_2 = 7.70 \pm 0.10$ ,  $n = 6$ ). Endothelium-dependent relaxant responses to acetylcholine were significantly attenuated after cryopreservation ( $E_{max} = 26\% \pm 5\%$ ,  $n = 4$ ) compared with unfrozen IMA ( $E_{max} = 71\% \pm 4\%$ ,  $n = 4$ ,  $p < 0.001$ ); endothelium-independent relaxant responses to sodium nitroprusside were unchanged.

**Conclusions:** Cryopreservation of human IMA under the conditions applied in this study (1) attenuated endothelial cell function and (2) induced an activation of protein kinase C, thereby increasing calcium influx through dihydropyridine-sensitive calcium channels. These experimental data suggest that postoperative administration of calcium channel blockers alone or combined with long-acting nitrates should effectively prevent the development of spasms in arterial grafts. (*J Vasc Surg* 1998;27:528-37.)

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Because of its superior long-term patency rate the human internal mammary artery (IMA) is increasingly used for autologous coronary bypass surgery.<sup>1</sup> However, a limited percentage of all patients undergoing coronary surgery require graft substitutes because of previous bypass surgery or other peripheral vascular disorders, and the search for alternative coronary grafts has been intensified. Cryopreserved homologous saphenous veins have been one of these alternatives for coronary artery bypass grafting. However, clinical observations suggested that these veins have poor angiographic patency.<sup>2</sup> Against this background the use of cadaveric cryopreserved IMA as coronary bypass conduits seems an attractive alternative. Recently, cryopreservation has also become an important tool for the storage of human vascular tissues in pharmacologic research.<sup>3-5</sup> These experiments have demonstrated that the postthaw functional recovery of cryopreserved isolated blood vessels is generally associated with reduced contractile force, endothelial function, and compliance.<sup>3,4</sup> Furthermore it has been shown that cryopreserved arteries may fracture during rapid thawing, but not if rewarmed slowly.<sup>6,7</sup>

To date it is largely unknown which alterations ultimately determine the net vascular function after cryopreserved blood vessels are rewarmed. Therefore the aim of our study was to investigate the consequences of various freezing/thawing protocols and whether the time of prefreezing exposure to the cryomedium could modify the postthaw functional activity of cryopreserved human IMA. In addition, changes in transmembrane signaling resulting from the freezing/thawing process were assessed with the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu).

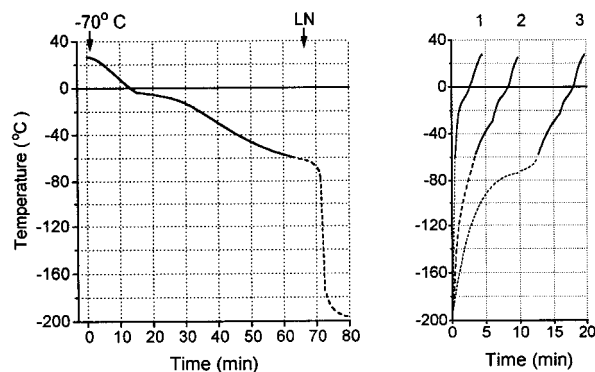
## MATERIAL AND METHODS

**Storage methods.** Internal mammary arteries (10 to 20 mm long, inner diameter  $\approx$  1 mm) were obtained during bypass surgery or were excised from multiple organ donors after permission was obtained from the local ethical committee. The preparations were placed in Krebs-Henseleit (KH) solution (composition mmol/L: NaCl 118, KCl 4.7, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.25, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, glucose 11, ethylenediamine tetraacetic acid 0.03) at room temperature and transported to the laboratory. The arteries were cleaned of loose connective tissue and used in organ bath studies either unfrozen within 20 hours after explantation or after cryopreservation. For cryopreservation the arterial segments were suspended in 2 ml liquid nitrogen storage ampoules

(Life Technologies AG, Basel, Switzerland) filled with KH solution containing 1.8 mol/L dimethyl sulphoxide (Me<sub>2</sub>SO) and 0.1 mol/L sucrose. The arteries were placed into the ampoules at different times so that the tissues were exposed to the cryomedium for 10, 60, 120, and 240 minutes at room temperature before being placed into a polystyrene box (5  $\times$  7  $\times$  15 cm) and slowly frozen at a mean cooling rate of approximately 1.3° C/min in a freezer maintained at -70° C. After approximately 2 hours or in rare cases on the next morning (after 20 hours at -70° C), the ampoules were transferred into liquid nitrogen (-196° C) to be stored until use. Before use the samples were thawed either rapidly at approximately 100° C/min by placing the ampoules for 4 minutes into a 40° C water bath (one-step thawing), or thawing was preceded by exposure for 6 minutes to room temperature (two-step thawing) at a mean thawing rate of approximately 30° C/min, or samples were first placed for 10 minutes on dry ice before being exposed for 6 minutes to room temperature and thawed within 4 minutes at 40° C (three-step thawing) at a mean thawing rate of approximately 15° C/min. Thereafter the preparations were rinsed in a dish containing 20 ml KH solution at room temperature to remove the cryoprotecting agents.

**Temperature measurements.** Temperature courses during freezing and thawing were measured with type du3s of Ellab a-s (Ellab Instruments, Copenhagen, Denmark). In these experiments the tip of a flexible thermoprobe was inserted into a 15 mm long segment of human IMA and fixed there by a cotton thread. The preparation was then frozen and thawed as described previously while temperature measurements were recorded digitally every 15 seconds.

**Organ bath studies.** After thawing was performed, the arterial segments were cut into rings (approximately 2 mm long), mounted between two hooks of stainless steel wire (diameter 0.15 mm), suspended in 10 ml organ baths containing KH solution at 37° C, and gassed continuously with 5% CO<sub>2</sub> in oxygen as described previously.<sup>4,8</sup> Changes in the tone of the preparations were recorded isometrically under a resting tension of 1 gm with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. At the beginning of the experiments the rings were stretched to an initial tension of approximately 2 gm and allowed to relax and equilibrate for approximately 2 to 3 hours in the bathing medium. During this time the vascular preparations were challenged once with nora-



**Fig. 1.** Temperature changes recorded within the lumen of a human IMA during freezing (**left**) and thawing at various procedures (**right**) while suspended in KH solution containing 1.8 mol/L  $\text{Me}_2\text{SO}$  and 0.1 mol/L sucrose. Freezing was performed as described in Methods. For thawing samples were removed from liquid nitrogen (LN) and thawed either rapidly by placing ampoules for 4 minutes into 40° C water bath (1), or samples were first exposed for 6 minutes to room temperature followed by thawing within 4 minutes in 40° C water bath (2), or samples were placed for 10 minutes on dry ice before being exposed during 6 minutes to room temperature followed by 4-minute thawing in 40° C water bath (3). Each curve represents mean of four recordings (single lines), extrapolated to -196° C (dotted lines).

drenaline (1  $\mu\text{mol/L}$ ), and the baseline tension of the rings was readjusted to 1 gm if required. Concentration-response curves for agonists were determined by cumulative additions, the concentration being increased when the maximum effect had been produced by the previous concentration. Responses to contracting agonists were expressed in grams or as percentages of the maximal effects of the control curves. In these experiments the contractile response of each arterial ring to noradrenaline (3  $\mu\text{mol/L}$ ) or prostaglandin  $\text{F}_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ) (30  $\mu\text{mol/L}$ ) was first determined, and this effect was taken as a point of comparison for subsequent responses. During repeated changes of the bathing solution at 15-minute intervals the arteries were allowed to relax before cumulative concentration-response curves for the agonist under investigation were determined without and in the presence of the antagonist added 20 minutes before the first administration of the agonist. Experiments with dihydropyridine derivatives and phorbol 12,13-dibutyrate (PDBu) were performed in organ baths protected from light to avoid inactivation of the compounds. When responses to relaxant agonists were investigated, active tone was induced by the

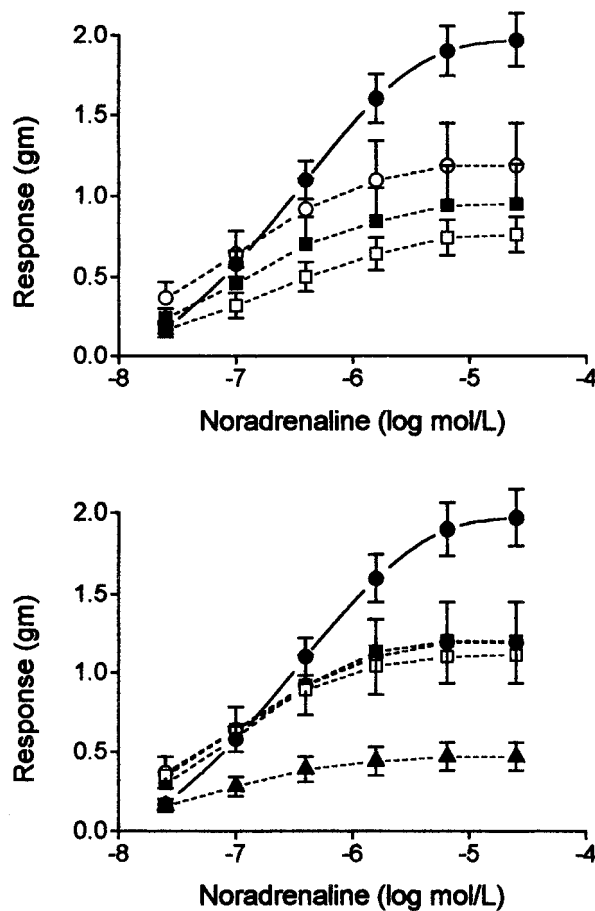
addition of 10 nmol/L U46619, which elicited a stable contraction of approximately 50% of its maximum. At the end of these concentration-response curves, papaverine (300  $\mu\text{mol/L}$ ) or sodium nitroprusside (100  $\mu\text{mol/L}$ ) was added to induce complete relaxation of the rings. This effect was taken as 100% relaxation.

**Drugs.** The following drugs were used: (-)-noradrenaline hydrogentartrate, acetylcholine chloride (Fluka, Buchs, Switzerland), indomethacin, nifedipine, PDBu (phorbol 12, 13-dibutyrate), sodium nitroprusside dihydrate, U46619 (9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxymethanoprostaglandin  $\text{F}_{2\alpha}$ ), staurosporine (Sigma, Munich, FRG), and  $\text{PGF}_{2\alpha}$  (Upjohn, Crawley, U.K.). The calcium channel opener 209-266 ((+)-(S) enantiomer of isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate) was synthesized as described previously at Sandoz Pharma Ltd. (Basel, Switzerland).<sup>9</sup> Nifedipine, 209-266, and U46619 were dissolved in ethanol to give a 1 mmol/L solution containing 60% ethanol. Staurosporine and PDBu were dissolved in  $\text{Me}_2\text{SO}$ . Samples of 0.1 mmol/L PDBu and 0.01 mmol/L staurosporine, each containing 1%  $\text{Me}_2\text{SO}$  and samples of 10  $\mu\text{mol/L}$  U46619, were stored at -20° C until use.

**Data analysis.** Concentration-response curves were analyzed with a computer program in RS/1 (BBN Software Products Corporation, Cambridge, Mass.), and  $\text{E}_{\text{max}}$  (maximal effects) and  $\text{pD}_2$  values (negative logarithm of the molar concentration of the agonists producing 50% of  $\text{E}_{\text{max}}$ ) were derived from this analysis. For insurmountable antagonism apparent  $\text{pD}'_2$  values (negative logarithm of the molar concentration of the antagonist reducing the maximum effect of the agonist by 50%) were calculated according to the equation  $\text{pD}'_2 = \text{pD}'_x + \log(x-1)$ , where x is the ratio of  $\text{E}_{\text{max}}$  of the agonist in the absence and presence of the antagonist and  $\text{pD}'_x$  is the negative logarithm of the molar concentration of the antagonist used. One-way analysis of variance (ANOVA) was performed, followed by the Bonferroni-corrected *t* test to assign differences to individual between-group comparisons when overall significance ( $p < 0.05$ ) was attained. Data are presented as mean  $\pm$  SEM.

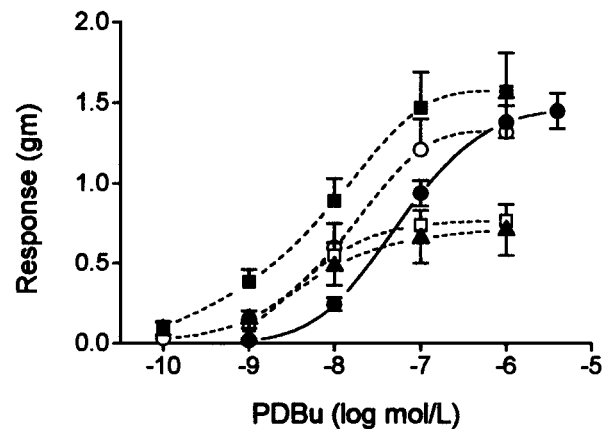
## RESULTS

**Thawing experiments.** A first series of experiments was performed to investigate the influence of three different thawing protocols on the postthaw contractile activity of arteries that had been frozen



**Fig. 2.** Concentration-response curves for noradrenaline on rings from human IMA without (*solid circles*,  $n = 50$ ) and after cryopreservation. Responses are expressed in grams; bars represent mean  $\pm$  SEM. **Top:** After 10-minute equilibration with cryomedium before cryopreservation, thawing was achieved within 4 minutes in 40° C water bath without (one-step, *empty squares*,  $n = 12$ ), after 6-minute exposure to room temperature (two-step, *solid squares*,  $n = 12$ ), or after 10-minute exposure to dry ice followed by 6-minute exposure to room temperature (three-step, *empty circles*,  $n = 12$ ). **Bottom:** Samples were cryopreserved after equilibration with cryomedium at room temperature for 10 minutes (*empty circles*,  $n = 12$ ), 60 minutes (*solid squares*,  $n = 30$ ), 120 minutes (*empty squares*,  $n = 12$ ), and 240 minutes (*solid triangles*,  $n = 12$ ) and thawed according to three-step thawing procedure.

after 10-minute equilibration with the cryomedium. Temperature changes during cooling and these three thawing protocols, recorded inside the lumen of a human IMA, are illustrated in Fig. 1. After thawing was performed, the maximal contractile responses to noradrenaline were generally diminished by 40% to 60% when compared with those of the unfrozen



**Fig. 3.** Concentration-response curves for PDBu on rings from human IMA without (*solid circles*,  $n = 32$ ) and after cryopreservation after equilibration with cryomedium at room temperature for 10 minutes (*empty circles*,  $n = 10$ ), 60 minutes (*solid squares*,  $n = 30$ ), 120 minutes (*empty squares*,  $n = 10$ ), and 240 minutes (*solid triangles*,  $n = 10$ ). Responses are expressed in grams; bars represent mean  $\pm$  SEM.

control group. Although the differences between maximal noradrenaline effects after the three thawing procedures were not significant, postthaw contractile responsiveness to noradrenaline of slowly thawed arteries appeared to be slightly better than in rapidly thawed tissues (Fig. 2., top, Table I). Therefore all samples used in the subsequent experiments were thawed slowly according to the three-step thawing protocol.

**Equilibration experiments.** In a second series of experiments the postthaw functional recovery of human IMA after different prefreezing equilibration times of the tissues with the cryomedium were investigated. Prefreezing equilibration with the cryomedium for 10, 60, and 120 minutes at room temperature reduced the maximal contractile responses to noradrenaline to approximately 60% of that produced by unfrozen arteries, and no differences were seen among these three groups. Equilibration for 240 minutes with the cryomedium before the freezing process was begun reduced the postthaw contractility markedly to 25% of that obtained with unfrozen tissues (Fig. 2, bottom, Table I). As indicated by the calculated  $pD_2$  values, noradrenaline consistently stimulated cryopreserved IMA at approximately two to three times lower concentrations than those of the unfrozen control group, suggesting a postthaw sensitization to  $\alpha$ -adrenoceptor-mediated contractile responses of the arterial smooth muscle. In contrast, neither efficacy nor

**Table I.** Postthaw parameters ( $E_{\max}$  and  $pD_2$  values) for contractile responses to noradrenaline and PDBu of rings from human internal mammary arteries after various thawing rates and prefreezing equilibration times with the cryomedium

Equilibration/thawing	Noradrenaline	n	PDBu	n
$E_{\max}$ (gm)				
Unfrozen IMA	1.97 ± 0.17	50	1.45 ± 0.11	32
10 min/one-step	0.76 ± 0.11*	12		
10 min/two-step	0.95 ± 0.25*	12		
10 min/three-step	1.19 ± 0.26	12	1.32 ± 0.21	10
60 min/three-step	1.20 ± 0.16*	30	1.57 ± 0.24	30
120 min/three-step	1.11 ± 0.20*	12	0.77 ± 0.10*†	10
240 min/three-step	0.47 ± 0.09*†	12	0.71 ± 0.16*†	10
$pD_2$ value				
Unfrozen IMA	6.55 ± 0.06	50	7.36 ± 0.07	32
10 min/one-step	6.79 ± 0.12	12		
10 min/two-step	6.98 ± 0.09*	12		
10 min/three-step	7.16 ± 0.08*	12	8.05 ± 0.17*	10
60 min/three-step	7.13 ± 0.06*	30	8.31 ± 0.09*	30
120 min/three-step	7.24 ± 0.07*	12	8.38 ± 0.08*	10
240 min/three-step	7.12 ± 0.09*	12	8.33 ± 0.06*	10

$pD_2$  value = negative logarithm of the molar concentration of an agonist producing 50% of maximum response.

\*Significant differences against values determined in unfrozen tissues.

†Significant differences against values determined in tissues frozen after 10-, 60-, and 120-minute equilibration and thawed according to the three-step protocol. Data are given as mean ± SEM.

**Table II.** Apparent  $pD'_2$  values for antagonism of PDBu and noradrenaline in rings from human internal mammary arteries cryopreserved after 60 minutes of prefreezing equilibration with the cryomedium and in unfrozen control arteries

	Cryopreserved IMA	n	Unfrozen IMA	n	Difference significant
PDBu - Staurosporine	7.65 ± 0.10	15	7.30 ± 0.08	12	$p < 0.01$
NA - Staurosporine	6.72 ± 0.09	13	6.74 ± 0.09	10	NS
NA - Nifedipine	7.75 ± 0.15	8	7.50 ± 0.10	6	NS

$pD'_2$  value = negative logarithm of the molar concentration of an antagonists that reduces the maximal effect of the agonist by 50%. Data are given as mean ± SEM.

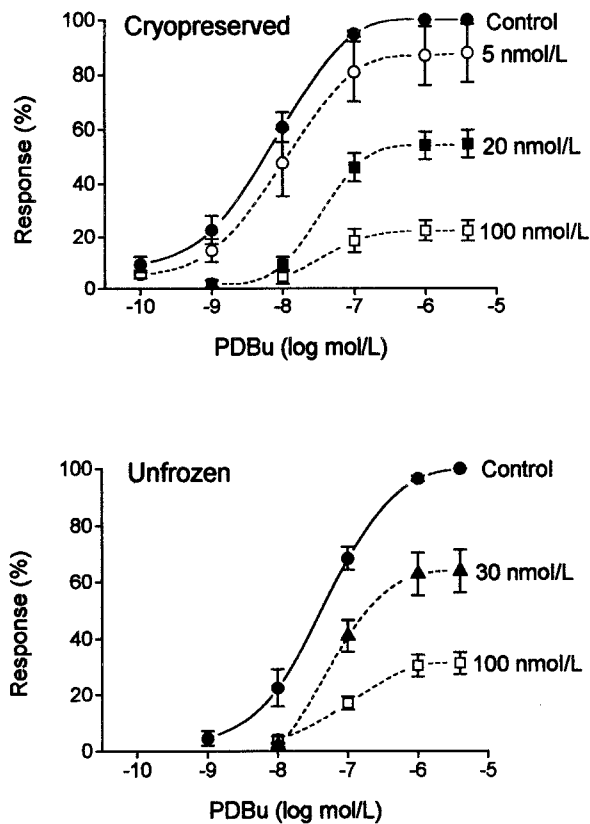
NA, Noradrenaline; NS, not significant.

potency of noradrenaline was changed if arterial smooth muscle preparations had been equilibrated at room temperature for 60 minutes with the cryomedium without freezing ( $n = 5$ , not illustrated).

When contractile responses of human IMA were elicited through direct activation of PKC by PDBu, maximal contractile responses to PDBu of arteries that had been frozen after 10 and 60 minutes equilibration with the cryomedium were similar to those of unfrozen control arteries. Only prolonged exposure for 120 and 240 minutes to the cryomedium before the cooling process was begun attenuated maximal contractile responses to PDBu. However, in all groups of cryopreserved IMA the concentration-response curves to PDBu were shifted to the left by a factor of 4 to 9 when compared with those

of unfrozen control tissues. Maximal sensitization to stimulation by PDBu was observed in arteries that had been equilibrated for 60 minutes with the cryomedium before the freezing process was started (Fig. 3, Table I).

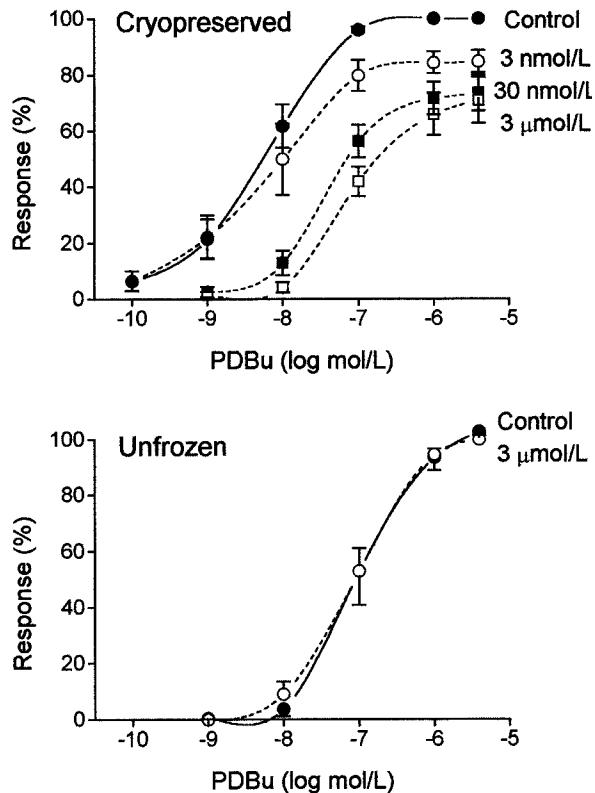
**Antagonism by staurosporine.** Experiments with antagonists were performed with tissues that had been frozen after 60-minute equilibration with the cryomedium, the data being compared with those determined on unfrozen arteries. In both unfrozen and cryopreserved arteries contractile responses to PDBu were attenuated when the PKC inhibitor staurosporine was present in the bathing fluid. Responses to PDBu of cryopreserved arteries (Fig. 4, top) were slightly but significantly more susceptible to inhibition by staurosporine than



**Fig. 4.** Concentration-response curves for PDBu on rings from human IMA cryopreserved after 60-minute equilibration with cryomedium at room temperature (top) and on unfrozen controls (bottom) without (solid circles) and in presence of staurosporine, 5 nmol/L (empty circles), 20 nmol/L (solid squares), 30 nmol/L (solid triangles), and 100 nmol/L (empty squares) added 20 minutes before agonist. Effects are expressed as percentages of controls; bars represent mean  $\pm$  SEM from five to six individual experiments.

unfrozen tissues (Fig. 4, bottom). As indicated by the calculated  $pD'_2$  values (Table II), approximately two times lower concentrations of staurosporine were sufficient to antagonize responses of cryopreserved IMA to PDBu to the same extent as in the unfrozen control group. When tested against noradrenaline, staurosporine proved to be equipotent in both cryopreserved and unfrozen arteries (Table II).

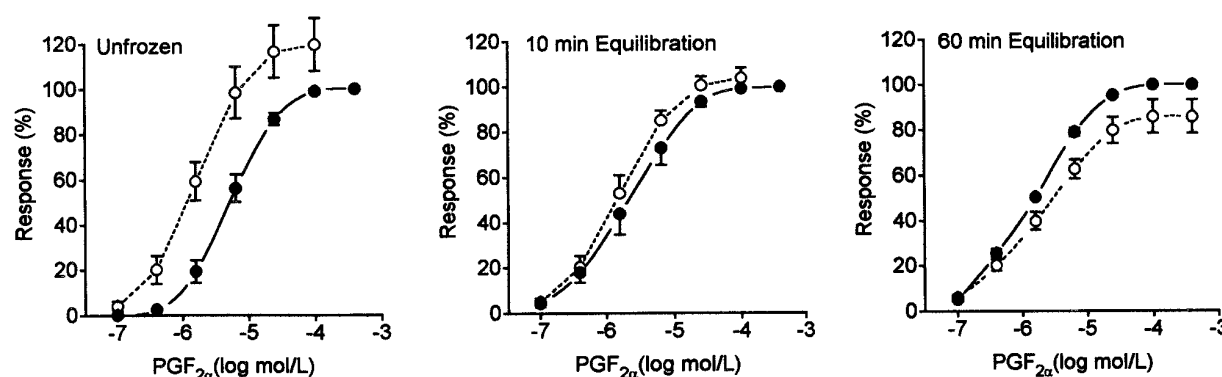
**Modification of calcium influx.** Contractile responses of cryopreserved IMA to PDBu proved to be highly susceptible to blockade of calcium influx by nifedipine. In the presence of 3 and 30 nmol/L nifedipine, the concentration-response curve to PDBu was shifted to the right in a concentration-dependent manner. However, a 100 times higher concentration of the calcium channel blocker (3



**Fig. 5.** Concentration-response curves for PDBu on rings from human IMA cryopreserved after 60-minute equilibration with cryomedium at room temperature (top) and on unfrozen controls (bottom) without (solid circles) and in presence of nifedipine, 3 nmol/L (empty circles), 30 nmol/L (solid squares), and 3  $\mu$ mol/L (empty squares) added 20 minutes before agonist. Effects are expressed as percentages of controls; bars represent mean  $\pm$  SEM from four to five individual experiments.

$\mu$ mol/L) elicited no further attenuation of the PDBu effects (Fig. 5, top). On cryopreserved IMA in the presence of 3  $\mu$ mol/L nifedipine, PDBu produced a  $pD_2$  value of  $7.17 \pm 0.03$  ( $n = 5$ ), which was not different from the  $pD_2$  value ( $7.07 \pm 0.15$ ,  $n = 4$ ) determined on unfrozen control arteries in this series. In contrast, the same nifedipine concentration (3  $\mu$ mol/L) did not modify responses to PDBu when tested on unfrozen human IMA (Fig. 5, bottom, Table III). When tested against noradrenaline, nifedipine (3 and 30 nmol/L) was equipotent on both cryopreserved and unfrozen IMA with similar  $pD'_2$  values (Table II).

When concentration-response curves for PDBu were determined in the presence of the calcium channel opener 209-266 (100 nmol/L), a signifi-



**Fig. 6.** Concentration-response curves for  $\text{PGF}_{2\alpha}$  on rings from human IMA, unfrozen (left) and cryopreserved after 10-minute (middle) and 60-minute equilibration with cryomedium (right) at room temperature. Responses to  $\text{PGF}_{2\alpha}$  were determined without (solid circles) and in presence of indomethacin,  $1 \mu\text{mol/L}$  (empty circles) added 20 minutes before agonist. Effects are expressed as percentages of controls; bars represent mean  $\pm$  SEM from five individual experiments.

**Table III.** Apparent  $\text{pD}_2$  values and maximal responses ( $E_{\text{max}}$ ) of PDBu, noradrenaline, and  $\text{PGF}_{2\alpha}$  without and in the presence of various antagonists on rings from human IMA cryopreserved after 60-minute preequilibration with the cryomedium and in unfrozen control arteries

	Antagonist conc.	Cryopreserved IMA		Unfrozen IMA	
		$\text{pD}_2$ value	$E_{\text{max}}$ (%)	$\text{pD}_2$ value	$E_{\text{max}}$ (%)
PDBu - Nifedipine	0	$8.37 \pm 0.16$	100 (8)	$7.07 \pm 0.15$	100 (4)
	3 nmol/L	$8.30 \pm 0.28$	$85 \pm 4$ (5)*		
	30 nmol/L	$7.47 \pm 0.10^*$	$74 \pm 6$ (5)*		
	3 $\mu\text{mol/L}$	$7.17 \pm 0.03^*$	$71 \pm 8$ (5)*	$6.99 \pm 0.20$	$103 \pm 1$ (4)
PDBu - 209-266	0	$8.06 \pm 0.13$	100 (5)	$7.23 \pm 0.08$	100 (8)
	100 nmol/L	$8.68 \pm 0.19^*$	$104 \pm 4$ (5)	$7.53 \pm 0.13^*$	$110 \pm 3$ (8)*
NA - 209-266	0	$7.62 \pm 0.10$	100 (3)	$6.65 \pm 0.26$	100 (5)
	100 nmol/L	$7.64 \pm 0.13$	$104 \pm 2$ (3)	$6.55 \pm 0.13$	$96 \pm 12$ (5)
$\text{PGF}_{2\alpha}$ - Indomethacin	0	$5.82 \pm 0.02$	100 (5)	$5.29 \pm 0.09$	100 (5)
	1 $\mu\text{mol/L}$	$5.74 \pm 0.10$	$80 \pm 6$ (5)	$5.79 \pm 0.09^*$	$117 \pm 12$ (5)

Data are presented as mean  $\pm$  SEM; number of determinations in brackets.

NA, Noradrenaline.

\*Significant differences from corresponding control values.

cant shift to the left occurred in the concentration-response in both cryopreserved and unfrozen arteries (Table III). In contrast, neither in unfrozen nor in cryopreserved IMA did 209-266 modify contractile responses to  $\alpha$ -adrenoceptor stimulation with noradrenaline (Table III).

**Blockade of endogenous prostaglandin synthesis.** To investigate whether the time of preequilibration exposure to the  $\text{Me}_2\text{SO}$ -containing medium would modify endogenously synthesized prostaglandins, concentration-response curves to  $\text{PGF}_{2\alpha}$  were determined without and after blockade of endogenous prostaglandin synthesis by indomethacin on unfrozen IMA and on arteries that had been cryopreserved after 10 and 60 minutes of equi-

libration with the cryomedium (Fig. 6). After blockade of the endogenous prostaglandin synthesis was achieved with  $1 \mu\text{mol/L}$  indomethacin, responses to  $\text{PGF}_{2\alpha}$  of unfrozen IMA were enhanced but unchanged in cryopreserved arteries (Table III). Furthermore no statistical differences were seen between the calculated  $\text{pD}_2$  values for  $\text{PGF}_{2\alpha}$  in cryopreserved IMA and in unfrozen arteries after blockade of endogenous prostaglandin synthesis (Fig. 6, Table III).

**Relaxant responses.** Endothelium-independent relaxant responses to sodium nitroprusside, expressed as percentages of the maximal responses to  $300 \mu\text{mol/L}$  papaverine, occurred in cryopreserved human IMA ( $\text{pD}_2 = 6.83 \pm 0.07$ ,  $n = 8$ ) within the

same concentration range as observed in unfrozen control arteries ( $pD_2 = 6.74 \pm 0.06$ ,  $n = 28$ ). Moreover, postthaw maximal relaxant effects ( $E_{\max} = 92\% \pm 2\%$ ,  $n = 8$ ) were slightly but significantly enhanced when compared with those observed in unfrozen control tissues ( $E_{\max} = 79\% \pm 3\%$ ,  $n = 28$ ,  $p < 0.05$ ).

In contrast, endothelium-mediated relaxant responses to acetylcholine, expressed as percentages of the maximal responses to sodium nitroprusside, proved to be significantly ( $p < 0.001$ ) attenuated after cryopreservation. Compared with unfrozen IMA ( $E_{\max} = 71\% \pm 4\%$ ,  $n = 4$ ), maximal relaxation in response to acetylcholine was diminished by approximately 60% ( $E_{\max} = 26\% \pm 5\%$ ,  $n = 4$ ) when tested in arteries that had been cryopreserved after 60-minute equilibration with the cryomedium. In arteries that had been equilibrated with the cryomedium for 60 minutes at room temperature without subsequent freezing, the endothelium-dependent responses to acetylcholine were unchanged ( $n = 5$ , not illustrated).

## DISCUSSION

Recent experiments on rabbit carotid arteries have demonstrated that the clinically observed phenomenon of fractures in cryopreserved vascular grafts occurs during rapid rewarming, but not if thawing is performed slowly.<sup>6,7,10</sup> Therefore it was interesting whether the speed of thawing could also modify the postthaw functional activity of human arteries as assessed in pharmacologic testing. In this study thawing of cryopreserved human IMA at rates ranging from  $15^\circ \text{C}/\text{min}$  to  $100^\circ \text{C}/\text{min}$  had surprisingly little effect on the postthaw contractile activity, as indicated by a fairly similar displacement of the concentration-response curves to noradrenaline. It may be, however, that the predominantly circumferential location<sup>6</sup> of eventual fractures are of minor importance if contractions are recorded on arterial ring preparations, as was done in this study. Nevertheless, the data suggested that the postthaw contractile responsiveness to noradrenaline of slowly thawed arteries was slightly better than in rapidly thawed samples. Previous experiments with canine femoral arteries have demonstrated that prolonged prefreezing equilibration with a  $\text{Me}_2\text{SO}$ -containing medium reduces progressively maximal contractile responses to noradrenaline without changing the sensitivity to the catecholamine.<sup>8</sup> However, despite a generally diminished efficacy of noradrenaline, the present experiments on human IMA revealed a significant shift to the left of the noradrenaline curve in most cryopre-

served arteries, indicating increased sensitivity to  $\alpha$ -adrenoceptor stimulation after cryopreservation.

A significant shift to the left of the concentration-response curve to noradrenaline in human IMA has also been reported after mechanical removal of the endothelial cells.<sup>11,12</sup> It is well known that in human IMA endothelial cells contribute to the local control of vascular tone and modulate agonist-induced contractile responses of the arterial smooth muscle by secreting relaxing factors such as prostacyclin<sup>13</sup> and endothelium-derived relaxing factor(s).<sup>11,12,14-16</sup> Indeed, previous experiments on human mesenteric and coronary arteries have demonstrated that cryopreservation under the conditions used in this study induces considerable impairment of endothelial morphologic characteristics and function.<sup>4</sup> The marked attenuation of endothelium-mediated relaxant responses to acetylcholine in this study therefore suggested that the enhanced sensitivity to noradrenaline as indicated by a shift to the left of the concentration-response curve resulted from cryoinjury of the endothelial cell function. Further evidence for this result was presented by the finding that blockade of endogenous prostaglandin synthesis by indomethacin enhanced responses to  $\text{PGF}_{2\alpha}$  in unfrozen but not in cryopreserved arteries, thereby supporting the contention of a modulatory role of endothelium-secreted prostaglandins with relaxant activity in unfrozen human IMA.<sup>13</sup>

The presence of endothelium has also been associated with impaired  $^{45}\text{Ca}^{2+}$  influx through receptor operated calcium channels into the vascular smooth muscle, because in rat aorta removal of the endothelium enhances both noradrenaline-induced contractile responses and noradrenaline-dependent  $^{45}\text{Ca}^{2+}$  influx.<sup>17</sup> However, in this study the effects of noradrenaline were equally susceptible to inhibition of calcium influx by nifedipine in both unfrozen and cryopreserved tissues. Furthermore in both unfrozen and cryopreserved samples, responses to noradrenaline were unchanged in the presence of the calcium channel opener 209-266 at a concentration ( $100 \text{ nmol/L}$ ) that has been shown to enhance  $^{45}\text{Ca}^{2+}$  uptake and to promote contractile responses of canine saphenous veins to depolarization.<sup>18</sup> Hence, these data did not support the presumption that the modified postthaw sensitivity of cryopreserved IMA to noradrenaline resulted from cryoinjury of the endothelial cells.

Experiments with isolated canine femoral and coronary arteries have demonstrated that during stimulation with PDBu both release and action of



endothelium-derived relaxing factor(s) on the vascular smooth muscle are inhibited.<sup>19</sup> Therefore it might be possible that the postthaw attenuation of endothelium-dependent relaxant response to acetylcholine of human IMA in these experiments reflects activation of PKC induced by the freezing/thawing process. This mechanism could also explain the enhanced sensitivity to noradrenaline of frozen/thawed IMA.

Therefore further experiments were performed to investigate whether changes in transmembrane signaling mechanisms had contributed to the modified postthaw noradrenaline effects. Contractile responses to noradrenaline of human IMA are mediated predominantly through  $\alpha_1$ -adrenoceptors.<sup>15,20</sup> Stimulation of  $\alpha_1$ -adrenoceptors is thought to activate pertussis sensitive guanine nucleotide binding proteins (G proteins), leading to increased calcium influx and activation of phospholipase C. Phospholipase C then catalyzes the breakdown of phosphoinositide 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol, causing release of calcium from intracellular stores and activation of PKC, respectively.<sup>21</sup> Furthermore a negative feedback control of vascular contraction with activation of PKC may inhibit the formation of inositol 1,4,5-triphosphate.<sup>22</sup> Against this background activation of PKC could well explain the attenuated maximal responses to noradrenaline of cryopreserved IMA observed in this study. Indeed, phorbolsters have been reported to attenuate contractions of rat aorta in response to noradrenaline<sup>23</sup> and 5-hydroxytryptamine associated with inhibition of phosphoinositide turnover.<sup>24</sup>

PDBu stimulates PKC by acting at the same site as diacylglycerol.<sup>22,25</sup> Therefore PDBu was selected to assess the extent of activation of PKC in human IMA without and after cryopreservation. In arteries that had been equilibrated with the cryomedium for different times before freezing, a time-dependent shift to the left of the PDBu curve without reduction of the maximal contractile effects was observed. Maximal sensitization to PDBu-induced contractions was found in arteries that had been equilibrated for 60 minutes with the cryomedium before the cooling process was started. Therefore these arteries were used to investigate changes in transmembrane signaling after cryopreservation. Staurosporine, a potent though nonspecific inhibitor of PKC,<sup>26,27</sup> antagonized responses to PDBu in both cryopreserved and unfrozen IMA, being slightly more potent in cryopreserved tissues. Besides indicating that the contractile responses to PDBu were indeed mediated through PKC, these findings suggested an

activation of PKC by the cryopreservation process. Furthermore PDBu-induced contractile responses of cryopreserved arteries were highly susceptible to blockade of calcium entry, whereas in unfrozen tissues PDBu effects proved to be unequivocally resistant to the action of the calcium entry blocker nifedipine. In cryopreserved arteries, where the concentration-response curve of PDBu was markedly shifted to the left, nifedipine completely restored the PDBu curve, making it superimposable to that determined in unfrozen control arteries. Several studies on animal tissues support the contention that phorbolsters modulate dihydropyridine-sensitive calcium conductance through activation of PKC, thereby increasing transmembrane calcium influx,<sup>22,28-32</sup> presumably by protein phosphorylation of a calcium channel subunit.<sup>33</sup> Animal studies have also shown that activation of PKC may be initiated by exposure to a hypertonic solution such as elevated glucose concentration.<sup>30,34</sup> However, in our studies exposure of human IMA to the cryomedium without freezing provided no evidence for an activation of PKC. Therefore it may be hypothesized that in these experiments activation of PKC was induced by thermophysical stresses, because during freezing ice crystals will be dispersed throughout the tissue.

## CONCLUSION

The consensus of evidence suggests that activation of PKC associated with increased  $\text{Ca}^{2+}$  influx through dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels and reduced production of endogenous relaxing agents such as endothelium-derived relaxing factor(s) might be the underlying mechanism of spasms in cryopreserved vessel grafts. Therefore these data suggest that postoperative administration of calcium channel blocking agents should be an effective treatment to prevent the development of spasms in arterial grafts. Moreover, because postthaw vascular smooth muscle responses to the nitric oxide donor sodium nitroprusside were unchanged, the use of long-acting nitrates that relax arteries in an endothelium-independent manner may be useful as long as endothelial function is impaired. It remains to be determined how long after grafting these *in vitro* observed changes will persist and preventive treatment will be required. These aspects, however, can be evaluated only in clinical studies.

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